NOVEL REDOX CLAMPING AGENTS AND USES THEREOF

Field of the Invention

The present invention relates to a novel class of chemosensitizing agents that function by "locking" or
5 "clamping" cells at a selected cellular redox state. The selected cellular redox state in which the cells are maintained by these agents is optimal for the cellular processes mediating apoptosis, normal cellular growth control and differentiation. Accordingly, these agents are useful in
10 sensitizing cells to the effects of therapeutics including, but not limited to antineoplastics such as butyrate and butyrate analogs.

Background of the Invention

It is becoming increasingly clear that apoptosis plays
15 an important role in the promotional phase of cancer development. Suppression of apoptosis has been suggested to be a universal property of cancer promoters while a number of agents which upregulate apoptosis in pre-neoplastic and neoplastic cells have been suggested to be useful
20 therapeutically in the treatment of neoplasia (McCarty, M.F., *Med. Hypotheses*, 1998, 50(5):423-33).

For example, butyrate which has been shown to induce apoptosis in various types of cancer is currently being considered as a potential cancer chemotherapeutic agent.
25 The mechanism by which butyrate functions to induce apoptosis

and alter the growth and differentiation state of cancer cells is not known. However, treatment with butyrate has been shown to decrease cellular levels of reduced glutathione (GSH), a major determinant of cellular redox status (Benard, O., and
5   Balasubaramanian, K.A., *Mol. Cell Biochem.*, 1997, 170:109-114). This butyrate-induced decrease in cellular GSH levels correlates with the differentiation and apoptosis of cultured cancer cells. Furthermore, agents known to modulate (*i.e.,* decrease) cellular GSH levels also enhance the differentiating
10 effects of butyrate in short-term *in vitro* assays (Benard, O., and Balasubaramanian, K.A.,*Mol. Cell Biochem.*, 1997, 170:109-114). Butyrate derivatives have also been shown to exhibit radiosensitizing properties (Chung et al., *Radiat. Res.*, 1998, 149(2): 187-194). Generally, agents that increase the
15 sensitivity of cells to radiation do so by decreasing the natural ability of cells to oppose radiation-induced oxidative stress. Agents that decrease cellular glutathione or inhibit the action/expression of antioxidant enzymes/proteins sensitize cells to the effects of radiation. Therefore, the
20 radiosensitizing effects of butyrate reflect the ability of this agent to modify the redox capacity of cells.

However, while butyrate decreases cellular GSH, this short-chain fatty acid also induces genes that oppose this change in the cellular redox status. Specifically, butyrate
25 treatment is associated with the elevated expression of the potent antioxidant and metal-chelating protein, metallothionein (MT) (Liu et al., *In Vitro Cell Dev. Biol.*, 1992, 28A:320-326). Elevations in MT and other antioxidant-generating systems can oppose the redox conditions initially
30 attained by the effect of butyrate on GSH levels. The disruption of the cellular redox state by the effect of butyrate on cellular GSH levels, causes the cells to readjust the redox status by the modulation of redundant elements of redox control. Readjustments of the redox state in this
35 manner can oppose the therapeutic action of butyrate. For

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example, there is evidence that butyrate-induced MT expression can, in part, oppose the redox conditions initially attained by the effect of butyrate on GSH levels. There is evidence suggesting that butyrate-induced MT expression can, in part, 5 explain the gradual development of resistance to butyrate following prolonged treatment. Further, an over-expression of the antioxidant MT has been disclosed to compensate for low cellular GSH levels (Miura et al., *Life Sci.*, 1997 60(21):301-309).

10   A novel class of agents has now been identified with the ability to maintain cells in a selected redox state. These agents do not permit the cell to successfully compensate for treatment-induced alterations in cellular redox status. For this reason, these agents are useful in enhancing the 15 therapeutic activity of chemotherapeutic agents (such as butyrate) that is dependent upon the redox state of the cell. These agents are also useful in controlling hyperproliferation of cells and conditions associated with abnormal fluctuations in the redox state of cells.

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Summary of the Invention

A novel class of compounds, referred to herein as redox clamping agents, has now been identified which comprise thiol-containing molecules with the ability to reduce specific 25 cellular oxidizing systems and/or molecules while maintaining other redox active systems in the oxidized state so that a cell is maintained in a selected redox state.

Accordingly, an object of the present invention is to provide a method of maintaining cells in a selected redox 30 state which comprises contacting the cells with a redox clamping agent which maintains the cells in a selected redox state.

Another object of the present invention is to provide a method of sensitizing selected cells to a chemotherapeutic 35 agent known to induce a stress response in cells which

comprises   contacting   the   selected   cells   with   the
chemotherapeutic agent in combination with a redox clamping
agent.

Another object of the present invention is to provide
5 a method of treating cancer in a patient which comprises
administering to the patient a chemotherapeutic agent known
to induce a stress response in cancer cells in combination
with a redox clamping agent.

Another object of the present invention is to provide
10 a method of inhibiting hyperproliferation of cells which
comprises contacting the cells with a redox clamping agent.

Yet another object of the present invention is to
provide a method of stabilizing the redox state of cells with
abnormal fluctuations in their redox states which comprises
15 contacting the cells with a redox clamping agent.

Detailed Description of the Invention

The cellular redox status (*i.e.*, the capacity of cells
to conduct oxidation/reduction reactions and to maintain the
oxidized or reduced state of thiols) is an aspect of the
20 intracellular environment that has a controlling influence on
numerous cellular processes (Sun, Y., and Oberly, L.W., *Free
Rad. Biol. & Med.*, 1996, 21(3):335-348; Monteiro, H.P., and
Stern, A., *Free Rad. & Biol. & Med.*, 1996, 21(3): 323-333).
Redox sensitive pathways extend from simple metabolism to
25 critical cell signal transduction cascades controlling gene
expression, growth, differentiation and apoptosis.

A novel class of compounds has now been identified which
are capable of locking or clamping cells in a specific redox
state. These compounds, referred to herein as "redox clamping
30 agents" comprise thiol (sulfhydryl)-containing molecules with
specific chemical properties which include the ability to
reduce specific cellular oxidizing systems and/or molecules
while maintaining other redox active systems in the oxidized
state. This is a major differentiating aspect between these

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agents and other agents that affect cellular redox status. These redox clamping agents are distinct from antioxidants since antioxidants permit major swings in the redox status and can only prevent the development of a pro-oxidizing state.

5   The function of redox clamping agents is not solely to deplete cellular levels of antioxidants, thiols, or antioxidant proteins/enzymes (*i.e.*, metallothionein, glutathione reductase, etc.). Rather, the reason for the application of redox clamping agents to living systems is to 10 adjust and maintain the relative amounts of (redox defining) cellular constituents such that a specific redox condition is sustained. In this respect, redox clamping agents are distinct from agents that simply deplete cellular GSH or decrease the expression or cellular levels of antioxidant 15 proteins or enzymes. Unlike redox clamping agents, such agents do no oppose the readjustments of the redox state following treatment.

The ability of these agents to clamp or fix cells in a selected redox state renders these agents useful in a variety 20 of therapeutic applications. Redox clamping agents can be used in combination with numerous chemotherapies for the treatment of cancer and pre-neoplastic disease as well as other medical conditions characterized by hyperproliferation of cells or abnormal cellular redox states.

25   For example, butyrate shows promise for the clinical management of prostate cancer and benign prostate conditions. However, the serum levels required to achieve favorable results are high (> 5 mM) and difficult to maintain (Newmark, H.L., and Young, C.W., *J. Cell Biochem. Suppl.*, 1995, 22:247- 30 253). Furthermore, butyrate resistant cells can arise from tumors following prolonged treatment with this agent (Ho et al., *J. Cell Physiol.*, 1994, 160(2):213-226). Thus, there is a need for agents that can chemosensitize cells to butyrate and prevent the development of butyrate resistance. These 35 agents would serve to lower the serum levels of butyrate

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required for anti-tumor activity and extend the therapeutic period.

It is believed that the anti-tumor effects of butyrate are sensitive to and/or dependent upon cellular redox conditions. It has now been demonstrated that redox clamping agents of the present invention work to enhance the ability of butyrate to inhibit growth and induce the differentiation and apoptosis of established prostate cancer cell lines (i.e., LNCaP and PC-3).

Thirty redox modulating agents or cell culture conditions were tested for their ability to influence butyrate-mediated apoptosis. In addition, the effect of some of these agents or conditions on the ability of butyrate to decrease cellular GSH levels and increase cellular MT levels was examined. The focus of these studies was to identify agents that permit/promote the GSH-lowering effects of butyrate and oppose treatment-induced readjustment of redox status by the cell. Included in this screen were agents that are classical redox modulating agents. Other agents were chosen based on their ability to alter cellular redox states as determined in preliminary studies. A partial list of the tested agents is provided is the following table.

Group 1 Classical Redox Modulators Tested	Group 2 Additional Redox Modulators Tested	Group 3 Butyrate-sensitizing Redox Clamping Agents
Buthionine sulfoximine*	Cysteamine (2-aminoethanethiol)	DMSA (meso-2,3-dimercaptosuccinic acid)
Methionine sulfoximine	Lipoic acid (a.k.a. thioctic acid)	MESNA (2-mercaptoethane-sulfonic acid)
Ethacrynic acid (GST Inhibitor)	PDTC (pyrrolidine dithiocarbamate)	Reduced (i.e. 1/4) cystine in medium
NAC (N-acetyl cysteine)	2-Mercapto-1-propanesulfonic acid	

Acivacin*	2,3-Dimercaptor-1-propane-sulfonic acid*	
Propargyl-glycine*	DTT (dithiothreitol)	
Ascorbic acid*	Glutathione ester	

5   Although many of the agents listed above were found to enhance
butyrate-induced apoptosis (*), extended treatment with some
of the compounds resulted in cells having elevated levels of
GSH and/or MT.   Further, many of these agents were found to
be toxic to cells while others inhibited the ability of
10 butyrate to induce apoptosis in LNCaP cells.   However, two
compounds were identified that functioned in combination with
butyrate to induce apoptosis in LNCaP and PC-3 cells.

        One of the compounds found to function in combination
with butyrate was meso-2, 3-dimercaptosuccinic acid (DMSA).
15 This agent is currently used in the clinic to lower the body
burden of toxic, heavy metals following inadvertent human
exposure (Miller, A.L., *Altern. Med. Rev.*, 1998, 3(3):199-
207).   DMSA functions in this capacity by chelating heavy
metals that are deposited within tissues and facilitating
20 their excretion.   Although DMSA alone was found to be a weak
inducer of metallothionein (MT) in these studies, LNCaP cells
treated with combinations of DMSA and butyrate exhibited
levels of MT that were 75% lower than cells treated with
butyrate alone.   Therefore, DMSA attenuates the ability of
25 butyrate to elevate cellular MT levels.   The half-life of the
MT protein is dependent on the presence of transitional metal
ions such as zinc.   For example, it has been shown that MT-
metal complexes exhibit a resistance to protease digestion
(McKim et al., *Toxicol. Appl. Pharmacol.*, 1992, 116(19):117-
30 124).   Therefore, DMSA may function to decrease butyrate-
induced elevations in cellular MT levels by sequestering the
metal ions needed to stabilize the MT protein.

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The second compound found to function in combination with butyrate to induce apoptosis was 2-mercaptoethanesulfonic acid (MESNA). This finding was unexpected since MESNA is used clinically as a uroprotectant with putative antioxidant
5 properties (Holoye et al., *Am. J. Clin. Oncol.*, 1990, 13(2):148-155). However, while MESNA treatment alone was found to have minimal effect on apoptosis, coadministration of MESNA markedly enhanced butyrate-induced apoptosis. Interestingly, this agent completely inhibits butyrate-induced
10 MT expression and slightly enhances butyrate-induced decreases in cellular GSH.

The combination of all three agents was found to be the most effective at promoting apoptosis in the LNCaP human prostate cancer cell line.
15   Based on these experiment, both DMSA and MESNA have been identified as members of the class of redox clamping agents of the present invention since both compounds appear to force butyrate-treated cells to maintain a specific redox state that is apparently optimal for promoting differentiation and
20 apoptosis. Results from these *in vitro* experiments will be confirmed *in vivo* in an established mouse model for prostrate cancer. It has been demonstrated that established human prostate cell lines when grafted into nude mice will achieve and maintain a redox state that closely resembles that of *in*
25 *situ* prostate cancer cells (Canada et al., *Biochem. Pharmacol.*, 1996, 51:87-90). Accordingly, pre-clinical evaluation of these agents using the nude mouse model of human prostate cancer will be performed.

It is expected that the combination of
30 butyrate/DMSA/MESNA will inhibit the growth of human prostate tumors in the nude mouse model relative to other test groups. Other combinations (*i.e.*, butyrate/DMSA, butyrate/MESNA) are also expected to be a more effective therapy than butyrate alone. Examination of the tumors from butyrate-treated
35 animals is expected to show an increase in PSA mRNA, a

decrease in Bcl-2/Bax ratio and a decreased proliferation of cells relative to tumors from untreated animals. These indicators, which reflect increased differentiation and decreased tumor growth, should improve in tumors from animals treated with the various combination therapies. Furthermore, these indicators should correlate with decreased cellular levels of GSH and MT. For example, tumors with the highest PSA mRNA levels, lowest Bcl-2/Bax ratio and lowest proliferation indexes should contain levels of cellular GSH, MT and other redox-defining molecules that fall within a specific range and that define a specific redox state. Histologic examination should find less mitotic figures, normal chromatin condensation and characteristics of differentiation of tumors isolated from animals treated with combinations relative to controls and animals treated with butyrate alone. Accordingly, not only will these studies provide insight into the manner by which DMSA and MESNA function and interact to enhance the anti-tumor activity of butyrate, but they will also provide a means to identify and develop additional redox clamping agents that function in a similar manner. Further, results from *in vivo* studies, along with data from *in vitro* experiments described herein, will provide insight into dosage regimens, effective concentrations, toxicity and the therapeutic potential of the butyrate chemosensitizers for use in the design of human clinical trials.

Thus, as demonstrated herein, in one embodiment, the redox clamping agents of the present invention are useful in the treatment of neoplasia in conjunction with a chemotherapeutic agent known to induce a stress response in cancer cells. An example of such a chemotherapeutic agent is butyrate for the treatment of prostate cancer. Cancer cells respond to the stress induced by the chemotherapeutic agent by upregulating various cellular processes that oppose the stress. The drug-induced stress response makes the cancer

cells that survive the initial stress more robust thereby enabling them to grow in a more uncontrolled manner and to become resistant to subsequent treatment with the same or different chemotherapeutic agents. In the present invention,

5 however, the redox clamping agent is co-administered with the chemotherapeutic agent known to induce a stress response in cancer cells. The redox clamping agent is administered in combination, either prior to and/or during and/or after the administration of the chemotherapeutic agent. The redox

10 clamping agent functions to oppose the treatment-associated stress response of the cancer cells, by adjusting the redox state of these cells to a region where cell death mechanisms remain active thereby making the cancer cells more sensitive to killing by the chemotherapeutic agent, increasing the

15 efficacy of the chemotherapeutic agent and inhibiting the development of resistance.

In another embodiment, the redox clamping agents are used as antiproliferative agent to be administered before an angioplastic procedure. Angioplasty is a medical procedure

20 that is conducted to open an obstructed blood vessel. This permits an increased blood flow to oxygen-deprived tissue. However, the physical trauma inflicted on the internal walls of the vessel during angioplasty, as well as the abrupt changes in the oxygen concentration in the vessel following

25 this procedure, induces a transient proliferation of the cells constituting the vessel. This proliferative response can be significant, resulting in a post-procedure thickening of the vessel and decreased blood flow. This proliferative response makes angioplastic procedures less effective. Redox clamping

30 agents can be administered prior to and/or during and/or after and angioplastic procedure. These agents induce a redox state of the blood vessel cells that is not consistent with proliferation thereby minimizing the post-procedure proliferation of cells and making the angioplastic procedure

35 more effective.

The redox clamping agents can also be used to treat hyperproliferative conditions of the skin. An increased proliferation and/or altered differentiation of epidermal cells in various skin conditions (*i.e.*, psoriasis) is

5 associated with major alterations in the redox state of the cells. Similarly, elevated cellular levels of the antioxidant glutathione is observed in skin cells following exposure to sunlight (UV light). This alteration in the redox state is a major promotor of cell proliferation in the skin. Redox

10 clamping agents taken orally or included in a topic ointment lessen cell proliferation and normalize the differentiative process.

In yet another embodiment, these redox clamping agents are used for the treatment of Type II (adult onset) diabetes.

15 In Type II diabetes, the cellular process of basal and insulin induced uptake of glucose from the serum is altered. Glucose uptake by cells is mediated by glucose transporters. In diabetic individuals the expression/function of specific types of glucose transporters is decreased. This contributes to the

20 inability of individuals suffering from diabetes to clear serum glucose levels after and between meals. The expression of these transporters is known to be uniquely affected by the redox state of the cells. Dramatic fluctuations in the redox state of tissue of diabetics (due to diminished glucose uptake

25 and utilization) tend to down-regulate the expression and function of the glucose transporters. Such a deficit exacerbates the diabetic condition and promotes elevations in serum glucose. Treatment of the Type II diabetic with a redox clamping agent of the present invention stabilizes the redox

30 state of the cells and normalizes glucose transporter expression/function. This treatment permits individuals suffering from diabetes to more easily manage the disease by normalizing serum glucose levels, thereby slowing progression of this disease.

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The following nonlimiting examples are provided to further illustrate the present invention

EXAMPLES

Example 1: Effect of agents/combinations on apoptosis of

5 **LNCaP cells**

LNCaP cells were treated with the agents (alone or in combination) for 48 hours. In these studies, the concentrations used were: MESNA, 50 μM; DMSA, 50 μM; butyrate, 5 mM. The cells were then harvested and the percentage of

10 apoptotic cells appearing in the sub-diploid peak was determined flow cytometrically.

Example 2: Effect of agents on cellular reduced glutathione

(GSH) levels

Species-specific limitations of the method of GSH

15 determination precluded the use of human LNCaP cells for these studies as the method of GSH detection depends on an isoform of glutathione-S-transferase that is not prevalent in human cells to conjugate the dye to GSH resulting in a fluorescent product. Therefore, the effect of butyrate, MESNA and DMSA

20 on cellular GSH levels was investigated using the H4 rat hepatoma cell line. The GSH-specific fluorescence of butyrate-treated cells was found to be lower than control. Furthermore, DMSA and MESNA treatment decreased basal GSH levels, while other redox modulating agents (*i.e.*, lipoic

25 acid) increased cellular GSH levels. This is of interest since DMSA and MESNA enhanced butyrate-induced apoptosis while lipoic acid was found to be a potent inhibitor. In additional studies, a different cytometry-based method will be employed to determine the cellular levels of reduced glutathione in the

30 human prostate tumor cells. An HPLC-based method will also be used to quantify both reduced and oxidized glutathione.

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Example 3:   Effects of MESNA on cellular MT levels

LNCaP cells were treated as described in Example 1.
However, the cells were harvested at the 24 hour time period.
The cells were then fixed (paraformaldehyde), stained with a
5 metallothionein-specific (fluorescence-conjugated) antibody
and analyzed flow cytometrically. These data indicate that
both DMSA and MESNA can inhibit butyrate-induced increase in
cellular MT levels. This observation is consistent with the
characterization of DMSA and MESNA as redox clamping agents:
10 they appear to oppose the attempt of butyrate-treated cells
to become pro-reducing by inhibiting the expressing of
antioxidant proteins such as MT while maintaining/enhancing
the low GSH levels induced by butyrate.

Example 4:   In vivo model for prostate cancer

15   The animal model that will be used to test the effect
of butyrate combination therapies on human prostate cancer
involves injecting (s.c./flank) male athymic (nude) mice with
human prostate cancer cells (LNCaP) as described by Raffo et
al., *Cancer Res.*, 1995, 55:4438-4445. This method has been
20 shown to yield 100% prostate tumor establishment at the site
of injection in approximately 3 weeks. Following a brief (4
day) post-injection period, the mice will be randomly assigned
to a treatment group (see Table 2) where they will receive
treatment with one or more of the described agents.
25 Approximately forty (40) days post-injection, tumor
development will be measured (13) and the tumors will be
excised and processed to characterize the effect of the
therapeutic combinations on various aspects of tumor growth
and differentiation (see Table 2).
30   It is estimated that at least 15 mice/group will be used
in the study in accordance with principals set forth by Steel
and Torri (1980, Principals and Procedures of Statistics: A
Biometrical Approach. New York:McGraw-Hill Book Company, Inc).
Such estimates assume a pooled standard deviation of tumor

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volume of 0.8 and the minimal expected difference between
control and test groups of 40%.

Example 5: Administration of therapeutic agents to mice

5   DMSA and MESNA will be administered to the mice orally
in the drinking water. The pharmacokinetics of oral DMSA and
MESNA have been extensively characterized in humans (Aposhian,
H.V., and Aposhian, M.M., *Annu. Rev. Pharmacol. Toxicol.*,
1990, 30:279-306; and James et al., *Br. J. Clin. Pharmacol.*,
10 1987, 23(5):561-568). Stability studies indicate that both
DMSA and MESNA are stable in simple liquid and solid
preparations. Two dosage levels for DMSA and MESNA (*i.e.*, low
and high) will be used. The "low dose" will be determined by
extrapolation using effective doses in humans.   The "high
15 dose" will be threefold higher than the "low dose". Butyrate
will be administered to specific dosage groups in the form of
the orally active prodrug, tributyrin.   Pharmacokinetic data
indicate that an oral dose of 5-10 g tributyrin/Kg will result
in peak plasma butyrate levels of 1-2 mM (Yuan et al., *Proc.*
20 *Annu. Meet. Am. Assoc. Cancer Res.*, 1994, 35:A2556). This
serum concentration range has been shown to significantly
reduce LNCaP tumor growth in nude mice treated with another
butyrate prodrug, isobutyramide (Gleave et al., *J. Cell.*
Biochem., 1998, 69(3):271-281.).   Food/water intake of the
25 mice will be monitored. If intake varies significantly in any
treatment group relative to control or other test groups then
paired feeding may be implemented.   The fourteen proposed
treatment groups are listed in the table below.

Table 2:

Treatment Groups (>15 mice/group)	
Control	Tributyrin (TB)
DMSA (low)	DMSA (low) + TB
DMSA (high)	DMSA (high) + TB
MESNA (low)	MESNA (low) + TB
MESNA (high)	MESNA (high) + TB
DMPS + MESNA (low/low)	DMPS + MESNA (low/low) + TB
DMPS + MESNA (high/high)	DMPS + MESNA (high/high) + TB

Example 6: Tumor processing

        Tumor volume will be calculated using standard methods
and the tumors will be excised. Pieces of the tumors will be
extracted for Western or Northern blot analysis. For flow
cytometric analysis, a single cell suspension will be
generated from pieces of the tumors using Protease XIV
according to established protocols. Various aspects of these
cells (*i.e.*, GSH, MT, proliferation index, etc.) will be
determined as described in the following examples. In
addition, sections of tumor will be preserved and processed
for histologic examination. The kidneys and liver from each
animal will be preserved for determining treatment-associated
organ pathology. Other organs will be preserved and examined
if indicators of specific toxicity are observed or suspected.

Example 7: Human prostate cancer cell lines

        The human prostate cancer cell line, LNCaP, will be used
in these animal studies. This cell line is an androgen-
sensitive prostate cancer cell line that has been used
extensively in animal models of human prostate cancer.
However, once optimal doses and combinations have been
determined for the LNCaP tumors, additional studies in other
tumor model systems can be conducted. Since the LNCaP cell

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line has been extensively characterized, standard biomarkers reflecting aspects of growth, apoptosis and differentiation have been determined. These biomarkers will be used in this study to determine the anti-tumor effects of the therapeutic
5 combinations, to gain insight into the mechanisms by which DMSA and MESNA function to enhance the effect of butyrate, and to identify additional redox clamping agents.

Example 8: Effect of the therapeutic combinations on
10   apoptosis of LNCaP tumors.

        Apoptosis of LNCaP cells, as well as butyrate-induced apoptosis of other cell types, is associated with alterations in the ratio of Bcl-2, Bcl-X and Bax. This mechanism of apoptosis is in contrast to apoptotic processes involving the
15 altered (decreased) expression/function of p53. By measuring the relative quantities of the Bcl-2 family of proteins in the cells of control and treated tumors using standard Western blotting techniques, the ability of the therapeutic combinations to affect mechanisms of apoptosis can be
20 characterized. The expression of other relevant cellular proteins which mediate/reflect or inhibit apoptotic processes and cell cycle arrest (i.e., p53, p21/waf1, p27Kip1, etc.) can also be characterized by either Western or Northern blot analysis to address unexpected treatment-associated phenomena.
25 Detection/quantitation of these proteins or mRNAs are determined using standardized techniques. The percentage of apoptotic cells in tumors from various treatment groups will be determined flow cytometrically using the standard TUNEL assay. For some tumors, the TUNEL assay will be performed
30 using tissue sections.

Example 9: Effect of the therapeutic combinations on
             differentiation of LNCaP tumors.

        A differentiation marker known as PSA (prostate-specific antigen) has been characterized for the LNCaP cell line.

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Increased expression of this protein has been associated with an increase in the differentiation state of this cell line as well as other human prostate cell lines and actual prostate tumors (Gleave et al., *J. Cell. Biochem.*, 1998, 69(3):271-

5   281.).   The expression of PSA mRNA in LNCaP tumors will be determined in this study by conventional Northern blot analysis. This information will be used to determine the effect of the treatment on the differentiation state of the human tumor.

10 **Example 10:   Effect of the therapeutic combinations on proliferation of LNCaP tumors**

Several hours prior to sacrifice, some mice in each treatment group will be injected with bromodeoxyuridine (BrdU). Incorporation of this thymidine analog into the DNA

15 of proliferating cells permits determination of the proliferation index of the tumor flow cytometrically. Human tumor cells can be resolved from contaminating mouse cells cytometrically, since the DNA-specific dye (*i.e.*, propidium iodide) which is used in the assay, stains cells on the basis

20 of total DNA content (human cells have significantly more genomic DNA than mouse cells). A decrease in the percentage of cells incorporating BrdU in tumors from mice receiving a specific combination of agents will be interpreted as a treatment-associated inhibition of tumor growth.   These

25 findings will be associated to other characteristics of the tumor (*i.e.*, size/volume, expression of differentiation markers, etc).

Example 11:   Effect of the therapeutic combinations on the redox status of LNCaP tumors

30   Determinants of differentiation, apoptosis and growth will be correlated with treatment-associated alterations in redox status of the tumor cells. Currently, the redox state

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is defined in relative terms based on the cellular levels of reduced glutathione and MT. Decreased MT expression and lower cellular reduced glutathione levels is expected in tumor cells of animals receiving combination therapy (*i.e.*, butyrate and 5 MESNA and/or DMSA) compared to those untreated or treated with butyrate alone. It is possible that elevated GSH and MT levels from butyrate-induced antioxidant rebound will be observed in the tumors from animals receiving butyrate alone relative to tumors from untreated animals based on our 10 prediction. For this analysis, levels of GSH and MT in tumor cells will be determined flow cytometrically and by Western blot analysis. In addition, an HPLC-based method to quantify cellular levels of oxidized and reduced glutathione will be employed.